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Means for inducing differentiation of supernumerary hair cells and Deiters' cells in the developing organ of Corti

The invention relates to means for inducing differentiation of supernumerary hair  
5 cells and Deiters' cells in the developing organ of Corti and their uses for treating deafness.

The mammalian organ of Corti has a highly organized tissue patterning mode of intricate sensory and non-sensory cells. Molecular cues that drive this strict  
10 arrangement remain broadly unknown. In the embryonic day 19 organs of Corti, it was shown that roscovitine, a chemical inhibitor of cyclin-dependent kinases, significantly increased the number of hair cells (HCs) and corresponding supporting cells (SCs) by triggering differentiation of precursor cells without interacting with cell proliferation. The effect of roscovitine was mimicked by other  
15 CDK1, 2, 5, 7, inhibitors, but not by CDK4/6 and MAP kinase pathway antagonists. Immunohistochemical analysis indicated that roscovitine specific intracellular targets, CDK1, 2, 5, 7 and ERK1/2 were expressed in the organ of Corti and especially in Hensen's cells. Affinity chromatography studies showed a tight correlation between the protein levels of CDK1, 2 and 5 with the rate of  
20 roscovitine-induced supernumerary cells in the organ of Corti. In addition, it was demonstrated that basal kinase activity was higher and more roscovitine sensitive at the specific developmental stages allowing the emergence of supernumerary cells. The effects of roscovitine required the Math1 transcription factor, as shown by the lack of effect of roscovitine on organs of Corti derived from Math1-null  
25 mice. These results showed that cyclin-dependent kinases are involved in the normal development of the organ of Corti and that, at least in E19 embryos, inhibition of CDKs is sufficient to trigger the differentiation of HCs and SCs, presumably from the Hensen's cells progenitors. The effects of roscovitine may be due either to the inhibition of phosphorylation (CDK1/2/5 inhibition) or

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inhibition of synthesis (CDK7 inhibition) of factors repressing differentiation into the HC phenotype.

Development of the organ of Corti, the auditory sense organ of mammals, involves the differentiation of sensory hair cells (HC) (inner and outer) and non  
5 sensory supporting cells (SC) including Deiters' cells, pillar cells, inner phalangeal cells, tectal cells and Hensen's cells (Figure 1). Each of these cell types has a distinct morphology that contributes to the complex structural and functional properties of the organ of Corti. Production of new HCs occurs throughout life in the auditory and vestibular sensory receptor of fishes and amphibians and in the  
10 vestibular receptors of birds. In contrast, ongoing production of auditory HCs during post-embryonic life does not appear to occur in avian auditory sensory epithelium, although HCs lost after sound trauma- or ototoxin-induced death are replaced.

In mammals, embryonic HCs and SCs proliferation within the sensory epithelia  
15 culminates between embryonic days 13 (E13) and 15 (E15) while neonatal HC production never occurs under normal conditions.

In higher eucaryotes, the commitment to cellular differentiation is controlled by series of highly scheduled events, involving sequential signals that regulate decisions of cell cycle exit, cell survival and the onset of differentiation  
20 process. The choice to remain into or to withdraw from the cell cycle and to differentiate is made during the G1 phase of the cell cycle by the interaction of G1 cyclins, cyclin-dependent kinases (CDKs) and endogenous CDK inhibitors (CKI). Two classes of these endogenous CKIs have been identified: the INK4 family specifically inhibiting cyclin D-associated CDKs (CDK4 and CDK6), and the  
25 Cip/Kip family including p21, p27, and p57, inhibiting all types of CDK/cyclin complexes.

Cip/Kip proteins are thought to be critical terminal effectors of the signal transduction pathways that control cell differentiation. p27<sup>KIP1</sup>, which specifically

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inhibits CDK2, 4 and 6, has been shown to be an essential mediator of oligodendrocyte terminal differentiation.

p27<sup>KIP1</sup> expression is induced in the primordial organ of Corti between E12 and E14, correlating at this stage with cell division arrest in HCs' and SCs' progenitors.

5 In wild-type animals, p27<sup>KIP1</sup> expression is down-regulated during subsequent HC differentiation, but persists at high levels in differentiated SCs of the mature organ of Corti. In mice with a targeted deletion of the p27<sup>KIP1</sup> gene, spontaneous supernumerary HCs and SCs are present in the postnatal organ of Corti.

10 Ectopic expression of CDK2, but not CDK4 or any of the cyclin, inhibits NGF-mediated neuronal differentiation of PC12 cells.

In addition, cell differentiation has been reported to correlate with the suppression or activation of kinase activity in several cell types including those of the neuronal, hematopoietic and myocytic lineages.

15 Taken together, it is clear that CDKs play a crucial ubiquitous role in the fine tuning of the balance between cell proliferation and differentiation, but the specific CDKs involved may vary between distinct cell phenotypes.

The inventors have investigated the function of cell cycle regulatory molecules in HC and SC differentiation of embryonic day 19 (E19) rat organs of Corti. The E19 rat organ of Corti is a well suited model of HC and SC  
20 differentiation since culturing these explants results in the spontaneous appearance of supernumerary HCs and corresponding SCs via differentiation of Hensen's cells in 10% of the explants regions.

The inventors have found that roscovitine, a selective inhibitor of the cyclin-dependent kinases CDK1, CDK2, CDK5, CDK7 and, to a lesser extent, of  
25 ERK1/ERK2, as well as the other kinase inhibitors, such as purine derivatives, indirubins, purvulanols, significantly increases the appearance of supernumerary HCs and corresponding SCs in E19 rat organ of Corti after 5 days in vitro (DIV). A rapid decline in the formation of supernumerary HCs and corresponding SCs in response to roscovitine is

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observed between E19 and P0. This lack of responsiveness to roscovitine is associated with the decline of the CDK1, 2 and 5 expression and CDK1/2 enzymatic activity. CDK1,2,5 and 7 are preferentially localized in Hensen's cells, the proposed progenitor cells from which HCs and SCs differentiate.

5 Altogether, these results show that pharmacological CDK inhibitors may have a direct inducing effect on the differentiation of HCs and corresponding SCs from Hensen's cells.

Deletion of the mouse basic helix-loop-helix (bHLH) transcription factor *Math1* causes failure of HCs generation in the cochlea. In addition, *Math1* has been  
10 shown to be specifically expressed in differentiating HCs at early embryonic stages and is absent from SCs and from cells outside the sensory epithelium in the cochlea.

These data support *Math1* as an essential factor in the control of HC differentiation. To determine whether expression of *Math1* was required for the  
15 roscovitine-induced appearance of supernumerary HC, the inventors used of cultured organs of Corti from *Math*<sup>-/-</sup> mice. In *Math1*-null mice, the treatment of E15.5 organs of Corti with roscovitine does not induce the appearance of HCs after 5DIV. However, in heterozygote mice,  $\beta$ -gal positive supernumerary HCs arose. Taken together, these results demonstrate that the induction of HCs by  
20 roscovitine likely recapitulates the developmental pattern of HC development which is drastically *Math1*-dependent.

The invention has then for object to provide means for treating pathologies associated with dysfunction of the development of the organ of Corti, particularly deafness.

25 It particularly relates to the use of at least one kinase inhibitor for making drugs for inducing differentiation of supernumerary hair cells and Deiters' cells in the developing organ of Corti .

Said kinase inhibitor(s) is (are) particularly a kinase inhibitor selected in the group comprising purine derivatives, such as disclosed in US patent 6 316 456,

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preferably the so-called roscovitine, indirubins and purvulanols, and the pharmaceutically acceptable acid addition salts thereof.

Pharmaceutically acceptable acid addition salts of said compounds are formed with organic or inorganic acids according to usual methods.

- 5 Suitable acids comprise acetic, ascorbic, maleic, phosphoric, salicylic and tartaric acids.

The drugs are administered to a patient in need thereof in an efficient amount and in association with a pharmaceutically acceptable carrier.

Said carrier may be solid or liquid depending on the administration form.

- 10 The medicaments can be administered in various forms : parenterally, rectally, topically, transdermally or orally. They are more particularly administered by the oral or injectable route.

For administration by the oral route, lozenges, compressed tablets, pills, tablets, capsules, drops, syrups, suspensions or emulsions, may be used. These

- 15 compositions advantageously comprise 100 to 1000 mg of active principle per dose unit, preferably 300 to 600 mg.

Other forms of administration include injectable solutions for the intravenous, subcutaneous or intramuscular route, formulated from sterile or sterilizable solutions. They can also be suspensions or emulsions.

- 20 These injectable forms comprise 100 to 1000 mg of said compound, or a pharmaceutically acceptable salt thereof, preferably 300 to 600 mg, per dose unit.

The invention also relates to a method for inducing differentiation of supernumerary hair cells and Deiters' cells in the developing organ of Corti

- 25 comprising administering to a patient in need thereof an efficient amount of at least one kinase inhibitor as above defined, or a pharmaceutically acceptable acid addition salts thereof, in association with a pharmaceutically acceptable carrier.

Other characteristics and advantages of the invention will be given hereinafter with reference to the figures which represent, respectively,

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- Figure 1: Schematic representation of the structure of the cultured organ of Corti: Cross-sectional representation (B) and corresponding surface view (A) of the sensory epithelium at E19 as observed in organotypic explant cultures. IHC, inner hair cell; O1-O3, outer hair cells; d1-d3, Deiters' cells; t1-t2, tectal cells; h1-h4, Hensen's cells; ut, undertectal cells; p, pillar cells; ipc, inner phalangeal cells; bc, border cells. Color codes of A refer to B cell subtypes.

- Figure 2: Hair cells and Deiters' cells development in E19 rat organs of Corti following 5 days of culture in control medium (A-F) or in the presence of 10 :M roscovitine (G-L): The expression patterns of Myosin VIIA (A,D,G,J) and Jagged1 (B,E,H,K) in control (A-F) and roscovitine treated (G-L) E19 rat organs of Corti at 5DIV was investigated by immunolabeling and confocal microscopy. A-C, D-F, G-I, J-L represent single microscopic fields visualized in z-sections stack by confocal imaging. Scale bars = 15: m for all panels.

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- Figure 3: Roscovitine triggers the appearance of supernumerary Hair cells: Organs of Corti from E19 rat embryos were cultured for 5 days in the absence (A, C) or presence (B, D) of 10 :M roscovitine. Explants were analysed by photonic microscopy after toluidine blue -staining of semi-thin sections (A, B) or by scanning electron microscopy (C, D). In controls (C) hair cells are arranged in three orderly rows for outer hair cells (OHC) and a single row for inner hair cells (IHC), showing uniform orientation of their stereociliary bundles. In roscovitine-treated explants (D) many supernumerary OHCs were present while the IHCs were barely visible due to regrowth of a tectorial membrane. Bar = 15 :m for all panels.

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- Figure 4: Roscovitine effects: dose-response (A), kinetics (B) and developmental stage specificity (C): (A) dose-response curve for roscovitine-induced supernumerary HCs in E19 cultured organ of Corti explants. Each data bar represents the mean length of supernumerary HCs regions for a minimum of 4

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explants per experiment. (B) The mean length of supernumerary HCs regions in control condition or in the presence of roscovitine (10 :M) was calculated as a function of time in culture with E19 rat organ of Corti explants. (C) Developmental stage dependence of roscovitine-induced supernumerary cells. Ten :M roscovitine  
 5 was added to rat organ of Corti explants dissected from stages E17, E19, P0, P2 and P4. The mean length of supernumerary HCs regions was monitored after 5 days of culture. Results were expressed as mean  $\pm$  sem (n = 5). Statistical significance was determined using a Student's *t*-test. \* =  $p < 0.05$  and \*\*\* =  $p < 0.001$ .

10 - Figure 5: Roscovitine does not interfere with cell proliferation which is absent in the organ of Corti: Confocal images of the expression pattern of Myosin VIIA (A,C), jagged1 (E,G) and cdk2 (I,K) double labeled with BrdU (B,C,F,G,J and K) in whole-mount explants of E19 cochleas after 5DIV showing that none of these markers co-localized with BrdU positive cells that were only located in connective  
 15 tissues. Z-series of the entire thickness of the explant were saved and recombined to produce a single image. (D,H,L): schematic drawings depicting a cross-sectional view of the cytoarchitectural structure of organs of Corti for each ligne of immunostaining. Red-colored cells represent alternatively myosin VIIA-positive cells (D), jagged 1-positive cells (H) and cdk2-positive cells (L); BM = basal  
 20 membrane; green cells represent the location of dividing cells (i.e. BrdU positive) that were all observed outside the sensory epithelium. Scale Bar= 15 :M for all panels.

- Figure 6: In situ immunohistochemical localization of CDK1, CDK2, CDK5, CDK7 and ERK1/2 in E19 rat organs of Corti: E19 rat organ of Corti sections  
 25 immunostained with anti-CDK1 (A,C), anti-CDK2 (D,F), anti-CDK5 (G,I), anti-CDK7 (J,L), anti-ERK1/2 (M,O) antibodies and phalloidin-TRITC ( $\beta$ -actin in B,C,E,F,H,I,K,L,N). Recapitulative table of immunopositive cell phenotypes for

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each protein (P). Brackets = HCs, p = Pillar cells, d = Deiters' cells, h = Hensen's cells, ct = connective tissue, act =  $\beta$ -actin.

- Figure 7: Kinases expression in E19 and P0 organs of Corti explants ex vivo or  
5 cultured with or without 10 :M roscovitine. Levels of protein expression for  
CDK1/2 (A), CDK2 (B), CDK5 (C), CDK7 (D), ERK1 (E) and ERK2 (F) were  
analysed by Western immunoblot analysis at E19 and P0 after affinity purification  
on p9<sup>CKShs1</sup> or aminopurvalanol (p95) beads. Organs of Corti were dissected from  
E19 and P0 rats and protein levels were quantified directly (ex vivo condition) or  
10 after 5DIV in the presence or absence of 10 :M roscovitine. Results were expressed  
as percentages of protein levels observed in E19 ex vivo explants.

- Figure 8: Effect of treatment with 10 :M roscovitine on CDK1/2 activity in E19  
and P0 cultured organs of Corti after 5DIV. Kinase assays were performed using  
15 histone H1 as a substrate in the presence of 15 :M ATP. Reactions were stopped by  
addition of 40 :l of Laemmli sample buffer. Samples were boiled and separated by  
10% SDS-PAGE. Gels were diced and subjected to autoradiography. Densitometric  
analysis was performed using Image Master 1D software.

20 - Figure 9: Math1 is necessary for hair cell production induced by roscovitine.  
Surface views at the middle turn of an E18 *Math1* <sup>+/+</sup> (A), an E15.5 *Math1*  <sup>$\beta$ -gal/ $\beta$ -gal</sup>  
(B) and an E15.5 *Math1* <sup>+/ $\beta$ -gal</sup> (C) cochleas in culture for 5DIV in the presence of  
roscovitine (10:M). Myosin VIIA immunostaining is represented in brown and  $\beta$ -  
gal staining is in blue. Bar = 15 :m.

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## EXPERIMENTAL PROCEDURES

### *Culture and treatments of organs of Corti*

Time-pregnant Sprague-Dawley rats or neonates were killed by euthanasia on gestational days 17 (E17), E19, or on postnatal days 0 (day of birth = P0), P2 and P4 with CO<sub>2</sub>. Both cochleae were dissected free from the calvaria with watchmaker forceps and under a stereomicroscope. Each organ of Corti was freed from surrounding tissues and explanted intact onto the surface of a sterile membrane (Millicell™, 12 mm, Millipore, Bedford, MA, USA) in minimum essential medium (MEM; Gibco, Gent, Belgium) into a 24-well culture plate (Nunc). Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Medium was renewed every 2 days. (R)-Roscovitine (Alexis Corporation, San Diego, USA) was dissolved as a stock solution in DMSO and added as indicated.

### 15 *Generation of Math1 knock-in mice*

*Math1* knockout mice were generated by replacing the *Math1* open reading frame (ORF) with the selectable marker PGKhpvt as previously described by Ben-Arie et al.(1). *Math1*<sup>Δ-gal/Δ-gal</sup> knock-in mice were generated by replacing the *Math1* ORF with a Δ-galactosidase (Δ-gal) reporter gene so that Δ-gal is expressed in all cells that express *Math1* (2).

### *LacZ staining and genotyping of embryos*

Whole-mount *lacZ* staining was carried out on cultured E15.5 organ of Corti explants as previously described (2;3). After 5DIV, explants were fixed in 4% paraformaldehyde for 20 minutes, washed thoroughly in PBS and equilibrate in X-gal staining buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02% NP40, and 0.01% sodium deoxycholate). Explants were stained overnight in X-gal buffer with X-gal (1 mg/ml) then post-fixed in 4% paraformaldehyde.

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Genotyping of tail DNA was performed using Southern analysis of *Eco*RI digested DNA and probes as previously described (1).

#### *Semithin sections*

- 5 Explants were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 45 min. Specimens were then post-fixed in 1% OsO<sub>4</sub> in the same cacodylate buffer for 30 min., dehydrated in graded ethanol concentrations and propylene oxide and embedded in epoxy resin (Agar Scientific Ltd, UK). Organ of Corti explants were sectioned parallel to the longitudinal axis of the HCs. Serial semi-  
10 thin sections, 1 :m-thick, were cut with a diamond knife, stained with toluidine blue (0.5%) and observed by light microscopy.

#### *Scanning electron microscopy*

- Explants for surface ultrastructural analysis were fixed in 2.5% glutaraldehyde in  
15 0.1 M sodium cacodylate (pH 7.2) for 2 hours, washed three times in cacodylate buffer, then postfixed for 1 hour with 1% osmium tetroxide. Explants were dehydrated in ascending concentrations of ethanol, critical-point dried from liquid CO<sub>2</sub> and sputter coated with gold. All material was examined in a JEOL JSM-840 scanning electron microscope operating at 20 kV.

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#### *Immunohistochemistry, immunocytochemistry, and confocal microscopy*

- Immunohistochemistry, immunocytochemistry and confocal microscopy were carried out as previously described (4;5). Whole-mount preparations or cryostat sections (10-20 :m) were fixed with either 4% paraformaldehyde (10 min. at 20°C)  
25 or methanol (5 min. at -20°C). Primary antibodies incubations were performed overnight at 4°C. Preparations were washed three times in PBS before incubating with a secondary antibody conjugated to alexa488, alexa568 or TRITC fluorophore for 1 hour at 20°C. Sequential double labeling experiments were done with a 4% paraformaldehyde fixation step in between each immunoreaction. Preparations

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were mounted on microscope slides and coverslipped with Fluoprep mounting medium (BioMérieux, Marcy l'Etoile, France) and imaged using a Bio-Rad MRC1024 laser scanning confocal microscope. To label cells in S phase in vitro, BrdU (10 :M, Sigma, USA) which is incorporated into replicating DNA, was added  
5 to the cultures for 2 or 5DIV prior staining.

The following primary antibodies were used: anti-myosin VIIa (1:200; Tama Hasson, University of California at San Diego, San Diego, CA, USA), anti-CDK1 (1:100, SC-54, Santa Cruz Inc., USA) anti-CDK2 (1:100, SC-163, Santa Cruz), anti-CDK5 (1:100, SC-173, Santa Cruz), anti-CDK7 (1:100, SC-529, Santa Cruz), anti-  
10 ERK1 (reactive with ERK1 and ERK2, 1:100, SC-94, Santacruz), anti-Jagged1 (1:100, SC-6011, Santa Cruz), anti-BrdU-FITC-conjugated antibody (1:3, Becton-Dickinson, USA). Omission of primary antibodies resulted in a complete loss of immunostaining.

15 *In situ detection of apoptosis: TUNEL labelling*

The apoptosis in E19 rat organ of Corti explants was looked for by staining the specimens cultured for 1, 2 or 5DIV with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method using the ApopTag fluorescent detection kit (Oncor, Gaithersburg, MD) as previously  
20 described (6).

*Dose-response experiments and temporal dependence*

Organs of Corti were dissected from E19 rats and maintained in culture. At the time of seeding, the culture medium contained roscovitine at 1.25, 2.5, 5, 10, 30  
25 or 100 :M. After 5DIV, cultures were fixed and immunostained for myosinVIIa and jagged1 as described above. In order to establish the time for optimal appearance of supernumerary HCs, E19 rat organs of Corti were treated with 10 :M roscovitine and the fraction of supernumerary zone was determined following fixation and immunocytochemistry for myosinVIIa of the explants at 2, 4, 5 and 6

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DIV. E17, E19, P0, P2 and P4 rat organs of Corti were dissected and cultured for 5DIV in the presence of 10 :M roscovitine. For each condition, the explants were fixed and the supernumerary zones were counted after myosin VIIa immunostaining.

5

#### *Quantification of HCs within the organ of Corti*

Quantitative analysis of supernumerary HCs production was obtained by counting the number of HCs per length and surface of sensory epithelium regions from the medium turn as previously described (5). If supernumerary cells were actually produced, the number of cells per unit of length should increase while the number of cells per surface unit should remain at the same level. After showing that new cells effectively arose in our cultures conditions, the extent of supernumerary HCs was determined by measuring the length of the supernumerary zone on images obtained from the confocal microscope. The total length of each organ of Corti explant was measured and the extent of the region with supernumerary HCs was expressed as percent of total length. These supernumerary regions were defined by the presence of more than four rows of HCs.

#### 20 *Western blot analysis*

Cultured organs of Corti from 10 cochleae for each experimental condition were lysed in homogenisation buffer containing of 60 mM  $\beta$ -glycerophosphate, 15 mM p-nitrophenyl phosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM  $\text{MgCl}_2$ , 1 mM DTT, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenyl phosphate, 10 :g/ml leupeptin, 10 :g/ml aprotinin, 10 :g/ml soybean trypsin inhibitor and 100 :M benzamidine. After sonication, insoluble material was removed by centrifugation at 12 000 g for 15 minutes at 4°C. Protein concentration was determined by the Bradford assay (Biorad). For crude extract analysis, proteins were heat denaturated in Laemmli sample buffer and resolved by 10 %

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SDS-PAGE (0.75 mm thick gels) followed by transfer on 0.1  $\mu$ m nitrocellulose filters. These were blocked with 5 % low fat milk in Tris-Buffered Saline-Tween-20 for 1 hour at room temperature, then incubated 1 hour with anti-PSTAIRE (1:3000, P7962, Sigma); anti-CDK5 (1:500, SC-173, Santa Cruz), anti-CDK7 (1:500, SC-529, SantaCurz) or anti-ERK1 (1:4000, M7927, Sigma), and analyzed by Enhanced Chemiluminescence (ECL, Amersham). Quantification of the results was carried out by densitometric analysis using Image Master 1D (Amersham-pharmacia, Belgium).

#### 10 *Affinity matrices*

Purvalanol (NG-95) and p<sup>9CKShs1</sup> affinity matrices were synthesized as described (7;8;9;10). Just before use, 10  $\mu$ l of settled beads were washed in 1 ml of Bead Buffer (50 mM Tris pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml soybean trypsin inhibitor, 100  $\mu$ M benzamidine) and resuspended in 400  $\mu$ l of this buffer. Cell extracts prepared as described above were then added (1 mg total protein) and the tubes rotated at 4°C for 30 minutes. After a brief spin, the supernatant was removed and the beads were washed four times with 1 ml Bead Buffer before addition of 50  $\mu$ l of Laemmli sample buffer. Following heat denaturation for 3 minutes, the bound proteins were resolved by SDS-PAGE and analyzed by Western blotting as described above.

#### *Quantification of CDK activity*

p<sup>9CKShs1</sup>-sepharose affinity purification was also used to determine the histone H1 kinase activity of bound CDK1 and CDK2. After purification as described above, the p<sup>9CKShs1</sup>-sepharose kinases were incubated for 30 min at 30°C with 1  $\mu$ Ci [<sup>32</sup>P] ATP (1-3 Ci/mmol, Amersham) in the presence of 25  $\mu$ g histone H1 (Type III-S, Sigma) in a final volume of 30  $\mu$ l of buffer C (homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors). Assays were

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terminated by transferring the tube on ice. 30 :l 2x Laemmli sample buffer was added. Phosphorylation of the substrate was assessed by autoradiography after SDS-PAGE.

## 5 RESULTS

### *Induction of supernumerary hair cells by roscovitine and by other kinase inhibitors*

Cochlear explants from E19 rat embryos developed a normal sensory epithelium after 5 days in culture with a single row of inner hair cells and three rows of outer hair cells, both being specifically stained with myosin VIIa (Figure 2  
10 A, D). Inner and outer HCs are supported, respectively, by one row of inner phalangeal cells (IPC) and three rows of Deiters' cells, both specifically stained with anti-jagged1 antibodies as previously described (11) (Figure 1, Figure 2 B, E). Cultures treated during 5 days with 10 $\mu$ M roscovitine developed a higher number of supernumerary HCs and corresponding SCs (i.e. Deiters' cells and IPC)  
15 organized in rows as in control conditions (Figure 2G-L). The supernumerary regions were characterized by four to nine rows of outer HCs, two to three rows of inner HCs and six to twelve rows of the corresponding SCs. However, all the roscovitine-induced supernumerary HCs were still separated from each other by intervening SCs. The production of supernumerary HCs was confirmed with  
20 photonic microscopy of sections through the organ of Corti and with scanning electronic microscopic studies (Figure 3).

To ascertain whether the effect of roscovitine was mediated by the inhibition of specific kinases, a series of chemically related and unrelated CDK inhibitors (Table 1) was tested.

25 **Table 1. Induction of supernumerary HCs by various kinases inhibitors.** Compounds were tested at various concentrations for their ability to induce supernumerary HCs in isolated organs of Corti. The lowest concentration inducing maximum effect is presented. For inactive compounds the highest concentration tested is shown in parentheses. In addition to CDK inhibitors, the

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MAP kinase kinase inhibitor U0126 and the transcription inhibitors actinomycin D and DRB were also tested. The  $IC_{50}$  values of all compounds for CDK1/cyclin B inhibition is also provided. \* = maximum concentration tested.

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	Target	Concentration inducing maximum supernumerary HCs differentiation (:M)	CDK1 inhibition (IC <sub>50</sub> , :M) (Knockaert et al, 2002b)
<b>olomoucine</b>	CDK1, 2, 5 and 7 and MAPK pathway	30	7
<b>roscovitine</b>	CDK1, 2, 5 and 7 and MAPK pathway	10	0,45
<b>aminopurvalanol</b>	CDK1, 2, 5 and 7 and MAPK pathway	5	0,004
<b>iso-olomoucine</b>	non-effective analogue of olomoucine	no effect (100 :M)*	> 500
<b>6-methyl- aminopurvalanol</b> N6-methyl- aminopurvalanol	non-effective analogue of aminopurvalanol	no effect (100 :M)*	> 100
<b>alsterpauellone</b>	CDK1, 2, 5 and 7 and MAPK pathway	5	0,035
<b>indirubin-3'- monoxime</b>	CDK1, 2, 5 and 7	10	0,18
<b>fascaplysin</b>	CDK4 and 6	no effect (100 :M)*	no effect
<b>U0126</b>	MAPK pathway	no effect (100 :M)*	no effect
<b>Actinomycin D</b>	Transcription inhibition	0,0001 (toxic at higher concentrations)	??
<b>DRB</b>	Transcription inhibition	0,01 (toxic at higher concentrations)	??



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The 2,6,9 -tri-substituted purines were all able to induce the appearance of supernumerary HCs. Their efficiency in inducing this effect on isolated organs of Corti correlated with their potency in inhibiting CDKs (aminopurvalanol > roscovitine > olomoucine). The kinase inhibitors' inactive analogues, methyl-aminopurvalanol (8;10) and iso-olomoucine (12) had no effect on the isolated organs of Corti (Table 1). Other, chemically different, CDK1/2/5 inhibitors such as indirubin-3'-monoxime (13) and alsterpaullone (14) also induced the appearance of supernumerary HCs while the CDK4/6 inhibitor, fascaplysin (15) had no effect.

Since roscovitine, and other CDK inhibitory purines inhibit CDK7, that is involved in the activation of transcription, two inhibitors of transcription: 6-dichloro-1-beta-D-riboenzimidazole (DRB) and actinomycin D were tested(16;17). Both actinomycin D and DRB were able to induce supernumerary HCs (Table 1).

Roscovitine, and other CDK inhibitory purines have been shown to inhibit the MAP kinases ERK1 and ERK2 *in vitro* and *in vivo*. The MAPK pathway inhibitor, U0126 (18) was then used. Even at concentrations as high as 100 :M , U0126 did not induce supernumerary HCs (Table 1).

*Dose-dependence, time-course and developmental regulation of roscovitine-induced supernumerary HCs*

In order to rule out a possible rearrangement of the HCs in the organ of Corti due to a compression of the explant or to a migration of the sensory cells, we assessed the number of HCs per surface unit and length unit in control and roscovitine-treated explants (were assessed). The results showed that the density of HCs per surface unit in roscovitine-treated explants remained identical to that of control explants. These data clearly demonstrated that supernumerary HCs arose from an actual increase in number rather than from a rearrangement of the existing HCs. The length of E19 rat organs of Corti explants containing supernumerary HCs increased as the concentration of roscovitine was raised (Figure 4A). At 10 :M roscovitine and after 5DIV, supernumerary segments

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represented 52.9 %  $\pm$  4.3 (mean  $\pm$  S.D., n = 4 independent experiments) of the total length of the explants. At higher concentrations (30 and 100  $\mu$ M) roscovitine was found to be toxic for all cells in the organ of Corti. In contrast the average thickness (number of HCs rows) of supernumerary HCs segments was also significantly higher in roscovitine-treated vs. control explants.

The effect of roscovitine on the development of supernumerary HCs was directly related to the duration of treatment (Figure 4B). The average length of supernumerary zones in the E19 rat organ of Corti explants maintained in the presence of 10  $\mu$ M roscovitine increased progressively with time in culture, yielding a significant effect after 4DIV treatment, and a maximal effect subsequently to a 5DIV. The spontaneous occurrence of supernumerary HCs in cultured organotypic explants of organ of Corti decreased from E17 to P0 and this process totally vanished at P2 (figure 4C). Likewise, the effect of roscovitine on the induction of supernumerary HCs culminated at E17, slightly decreased at E19 and disappeared at P0.

*Mechanisms of roscovitine-induced supernumerary HCs does not rely on the regulation of cell proliferation and apoptotic cell death*

Roscovitine, like other CDK inhibitors, has potent anti-mitotic properties (reviewed in (19;20)). Cell proliferation in E19 explants of organ of Corti treated with roscovitine was then investigated. Explants were incubated for either 2 or 5 DIV in the presence of 10  $\mu$ M BrdU. No epithelial cells were labeled with BrdU in the area of HCs and SCs (Figure 5). Confocal analyses displayed mitotic figures that were observed exclusively in connective tissues of mesenchymal origin, while no BrdU positive cells were found within the planes of the sensory epithelium (figure 5). The rate of proliferation of mesenchymal cells located in connective tissue areas was actually higher in the absence of roscovitine consistent with its well established effect on cell cycle progression.

To determine whether apoptosis was implicated in HCs and SCs

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differentiation, E19 rat organ of Corti explants were double-stained for DNA fragmentation (TUNEL positive) and for f-actin in the stereocilia bundles and cuticular plate. After 1, 2 or 5 DIV, no TUNEL positive nuclei were identified either in control or roscovitine (10 :M) treated cultures (data not shown). The  
5 evidence that supernumerary HCs and corresponding SCs occur in the absence of cell division and apoptosis thus strongly supports the hypothesis of a direct differentiation of pre-existing immature progenitors cells into both HCs and corresponding SCs.

10 *Immunohistochemical localization of molecular targets of roscovitine in the E19 rat organ of Corti*

In order to characterize the molecular components of the transduction cascade underlying the effect of roscovitine on supernumerary cells, we first  
15 studied the expression of CDK1, CDK2, CDK5, CDK7 and ERK1/2 by immunohistochemistry using sections obtained from E19 rat organ of Corti (Figure 6). CDK1,CDK5, CDK7 and ERK1/2 were shown to be ubiquitously expressed throughout the organ of Corti, including the HCs (figure 6P). Interestingly, CDK2 was found to be specifically present in tectal cells, Hensen's  
20 cells (h in figure 6D-F) and to a lesser extent in pillar cells in E19 explants of organs of Corti. The specific expression pattern of CDK2 persisted throughout the early postnatal period (data not shown). It is important to emphasize that cells immuno-positive for myosin VIIA, i.e. HCs, or for jagged1, i.e. Deiters' cells and IPC, were never immunoreactive for CDK2 (data not shwon).

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*Expression of molecular targets of roscovitine in the organ of Corti ex vivo and in vitro*

The levels of expression of CDKs and ERKs following affinity purification (Figure 7)was then investigated and the levels of protein expression in organs of Corti were compared between two developmental stages, i.e. E19 and P0, which

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are respectively sensitive and insensitive to roscovitine for the induction of supernumerary HCs. E19 and P0 rat organs of Corti were dissected, lysed and CDK1, CDK2 and their associated proteins were purified by affinity chromatography on p9<sup>CKShs1</sup> beads, the mammalian homologue of CKS1 from *Saccharomyces cerevisiae* and p13<sup>suc1</sup> from *Schizosaccharomyces pombe*. The expression of CDK1/2 was assessed by immunoblotting analysis using PSTAIRE specific antibody while the expression of CDK2 alone was evaluated by a specific anti-CDK2 antibody. As shown in Figure 7A, the expression of CDK1/2 diminished between E19 and P0 for each condition (ex vivo explants and cultures with or without roscovitine). In contrast the level of CDK2 remained more constant (figure 7B) suggesting that the expression of CDK1 was lower at P0 ex vivo and that CDK1 was drastically down-regulated in vitro especially in the presence of roscovitine both with E19 and P0 organs of Corti.

Affinity chromatography on immobilized purvalanol which sensitizes the recovery of CDK5, CDK7, ERK1 and ERK2, that are also the specific targets of the 2,6,9-trisubstituted purines was used. The expression of CDK5 ex vivo was slightly higher at E19 ex vivo and increased in culture only in explants isolated from E19 organs of Corti both in control condition and in the presence of roscovitine. The expression of CDK5 diminished between E19 and P0 for each condition, i.e. explants and cultures in the presence or not of roscovitine (figure 7C). The expression of ERK1 and ERK2 was not different between E19 and P0 ex vivo and remained stable in culture (figure 7E and 7F). In contrast, CDK7 expression was much higher in P0 organs of Corti ex vivo (figure 7D).

#### 25 *Changes in CDK1/2 activities in E19 and P0 rat organs of Corti following roscovitine treatment*

The changes in CDK1/2 activities occurring in lysates of E19 and P0 organs of Corti during roscovitine-induced HCs and SCs differentiation (roscovitine 10

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:M) (Figure 8) was examined. p<sup>9CKShs1</sup>-sepharose affinity purified lysates were assessed for their ability to phosphorylate histone H1. As shown in Figure 8, kinase activities in control conditions was higher in cultures from E19 organs of Corti, consistent with data from Western blot analysis. Furthermore, in the presence of roscovitine (10 :M) a significant inhibition of CDK1/2 kinase activity that was relatively more important with E19 cultured organs of Corti was observed.

10 *Induction of supernumerary HCs and SCs by roscovitine requires the transcription factor Math1*

Further experiments were carried out to investigate whether the transcription factor Math1 was upstream or downstream of the roscovitine effect, using of the Math1 -/- mice (Bermingham et al, 1999). When organs of Corti dissected from E15 or E18 wild type mice were exposed to roscovitine for 5DIV myosin VIIA immunopositive supernumerary HCs, (Figure 9A) were observed. Myosin VIIA staining was absent in the cochlea of E15.5 *Math1*-null mutant mice (*Math1*<sup>Δgal/Δgal</sup>) cultured for 5DIV in control medium or in the presence of roscovitine (Figure 9B). In *Math1*<sup>+/-Δgal</sup> mice, it has been previously shown that Δgal was restricted to the HCs of the developing sensory epithelia (Bermingham et al., 1999). After culturing E15.5 *Math1*<sup>+/-Δgal</sup> organs of Corti for 5DIV in the presence of roscovitine at 10 :M, supernumerary HCs were present and identified by a myosin VIIA positive staining (figure 9C). These supernumerary myosin VIIA positive cells are also Δgal positive, i.e. *Math1* positive.

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## DISCUSSION

The mammalian organ of Corti is a one of a kind sensory epithelium characterized by a highly organized cytoarchitecture, with HCs and SCs arranged in a precise alternative pattern. Studying of how their production is controlled during development is crucial towards understanding the causes of deafness or attempting to induce HC regeneration. In rodents, auditory HCs and SCs precursors have their terminal mitosis between E13 and E17. However, a production of supernumerary HCs has been shown to occur after the ontogenesis of the sensory cells, in various circumstances. Previous studies reported the production of supernumerary HCs in embryonic cochlear explant cultures following retinoic acid treatment, in normal serum-free medium or in mice in which a specific gene is deleted, such as p27<sup>KIP1</sup> jagged-2, or HES transcription factors.

The results provide clear evidence that roscovitine and other pharmacological inhibitors of protein kinases induce the production of a large number of supernumerary HCs and corresponding SCs (i.e. Deiters' cells and IPC) in E19 rat organs of Corti.

### *Roscovitine-induced supernumerary HCs have all the features of preexisting HCs*

Evidence based on myosin VIIA staining and scanning electron microscopic observations showed that all HCs, including supernumerary HCs generated in the presence of roscovitine, shared the same phenotype in E19 rat organs of Corti explants cultured for 5 days. In addition, in supernumerary zones, the normal cytoarchitecture of the organ of Corti was conserved, as no area displaying only HCs without interposed SCs was never observed. Taken together, these results showed that progenitor cells which have retained the capacity to become HCs or corresponding SC can be induced by roscovitine to differentiate into newly formed cells according to a developmental program that likely recapitulates the events occurring during normal ontogenesis.

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*CDK inhibition triggers a phenotypic differentiation of precursor cells into supernumerary HCs and corresponding HCs without interacting with cell cycle progression and apoptosis*

The activity of CDKs critically contributes to the molecular switch between  
5 cell proliferation and differentiation in several cell types. Roscovitine has been described to induce cell differentiation through its inhibition of cell cycle progression leading to an arrest of proliferation. Such a mechanism is not involved in the production of supernumerary HCs and corresponding SCs because no cell proliferation was ever observed in cultured intact sensory epithelium from E19  
10 rats. Roscovitine has also been described as a pro-apoptotic agent in undifferentiated cells mostly by blocking the cell cycle or an anti-apoptotic agent on differentiated cells such as neurons.

TUNEL studies revealed no apoptotic cells within the sensory epithelium after 5 days of culture, both in control or roscovitine-treated conditions, excluding that a  
15 possible interaction of roscovitine with apoptotic processes may partly underlie the production of supernumerary HCs and corresponding SCs. The absence of proliferation and apoptosis in the presence of roscovitine within the organ of Corti argues in favor of a direct phenotypic conversion of pre-existing cells into HCs and corresponding SCs. Cell conversion has been observed in amphibian lateral  
20 line organ after laser ablation of HCs, in amphibian vestibular sensory epithelium after aminoglycoside-induced HC damage in avian basilar papilla after noise trauma or aminoglycoside damage and in utricular macula of mature guinea-pigs following gentamicin-induced HC death.

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*The targets of roscovitine in the organ of Corti*

Roscovitine is a pharmacological inhibitor of protein kinases which has been tested on over 25 kinases and possesses a high specificity for CDK1, 2, 5, 7

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and the MAP kinases ERK1 and ERK2 . Experiments were carried out to study whether the generation of supernumerary HCs and corresponding SCs in the organ of Corti was the result of the inhibition of any specific roscovitine's kinase targets. Roscovitine and other 2,6,9-tri-substituted purines, i.e. olomoucine and aminopurvalanol, are known to inhibit ERK1 and ERK2. However, U0126 which is a specific antagonist of the MAP Kinase pathway, did not induce the appearance of supernumerary cells. These data suggest that inhibition of ERK1/2 is unlikely to participate to the mechanism of action of roscovitine on the isolated organ of Corti. Fascaplysin, a CDK4 and CDK6 specific inhibitor, did not trigger the appearance of supernumerary cells. Furthermore, all other CDK inhibitors used in this study have no effect on CDK4, and yet triggered the appearance of supernumerary HCs and corresponding SCs (Table 1). Altogether this supports the view that CDK4/6 inhibition is not required for supernumerary HCs and corresponding SCs differentiation. Roscovitine analogues non-structurally related such as alsterpaullone and indirubin-3'-monoxime which are CDK1,2,5,7 inhibitors like roscovitine but devoid of effect on the MAP Kinase pathway also induced supernumerary HCs and corresponding SCs. Altogether, this demonstrates that roscovitine stimulates the differentiation of supernumerary cells by the inhibition of a subset of CDKs, i.e. CDK1, CDK2, CDK5 and CDK7.

*Hensen's cells, the roscovitine-sensitive HCs and corresponding SCs progenitor cells ?*

There is compelling evidence from both mammals and non-mammals that HCs and SCs share a common progenitor.

These models suggest that uncommitted cells within developing sensory epithelia essentially compete to become committed to the HC phenotype. Once an individual cell has become committed to differentiate as a HC, that cell then exerts a form of lateral inhibition on any neighboring cells that it contacts, so that the immediate neighbors of HCs are prevented from differentiating as HCs. Those inhibited cells are constrained to differentiate as Deiters cells. Indeed, in all in vitro and in vivo models, the appearance of supernumerary HCs is systematically



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accompanied by the production of supernumerary Deiters' cells or pillar cells while no overproduction of Hensen's cells have been described. The immunocytochemical data revealed that potential molecular targets of roscovitine, i.e. CDK1,2,5,7 are all ubiquitously expressed in the organ of Corti except CDK2  
5 which is only expressed in Hensen's cells and to a lesser extend in pillar cells. Previous works demonstrated the role of Hensen's cells as possible progenitors for HCs and corresponding SCs. All together these results suggest that Hensen's cells are suited candidates to represent the cellular source of roscovitine-induced supernumerary HCs and corresponding SCs.

10

*Molecular insights from developmental regulation of roscovitine-induced supernumerary HCs and corresponding SCs*

In order to untangle respective roles of CDK1,2,5,7 in the transduction pathway underlying the induction of supernumerary cells in the organ of Corti by  
15 roscovitine, levels of protein expression between two developmental stages that were permissive (E19) or repressive (P0) with respect to this phenomenon was compared. Moreover, not only spontaneous supernumerary cells were more seldom at P0 than at E19 but the stimulation of this process by roscovitine was absent at P0 and sustained a high level at E19. At different extent, CDK1,2 and 5  
20 expression were decreased at P0 ex vivo, consistent with the lower rate of emergence of supernumerary cells and with the absence of effect of roscovitine at this stage. The fact that transcription inhibitors such as actinomycin D and DRB also induce the apparition of supernumerary HCs and corresponding supporting cells, strongly suggest that inhibition of CDK7 could be involved.

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Altogether these results indicates that CDK1,2,5 and 7 are good candidates to mediate roscovitine effect on HCs and corresponding SCs differentiation.

Said results also demonstrate that CDK1/2 kinase activity was higher at E19 than at P0 and was actively inhibited by roscovitine at both stages but more severely at E19. The confirmation that roscovitine inhibited CDK enzymatic

activity validated that this pharmacological agent was indeed exerting its normal biochemical properties in our in vitro model of organotypic cultures of organ of Corti.

5     *Math1 acts downstream of roscovitine*

*Math1*, a bHLH proneural gene appear necessary for the production of HCs induced by roscovitine since in organ of Corti explants from *Math1*<sup>Δgal/Δgal</sup> no HCs are produced in the presence of Roscovitine. In addition in heterozygote mice, *Math1* is upregulated in supernumerary HCs generated by roscovitine. These  
10     results confirm previous reports showing that *Math1* is necessary and sufficient for the differentiation of HCs in the mammals cochlea (Bermingham et al., 1999;Zheng and Gao, 2000). They demonstrate that roscovitine requires the downstream intervention of Math1 for its differentiating effect.

15             In conclusion, the invention demonstrates that CDKs play a role in cellular differentiation in the organ the Corti.

          Roscovitine was regularly administered to patients suffering of deafness. An improvement of their troubles was observed without any drawbacks.

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